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1 **Pipecolate and taurine rat urinary biomarkers for lysine and threonine deficiency**
2 **respectively**

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20 **Running title:** Biomarkers of lysine and threonine deficiency.

21 **Abbreviations:** EAA: essential amino acid, IC-DA: Independent Component - Discriminant
22 Analysis, L: Lysine, LC-MS: Liquid Chromatography-Mass Spectrometry, P: Proteins, PLS:
23 Partial Least Squares, PLT: Total Milk Protein, T: Threonine.

24 **Abstract**

25 **Background:** The consumption of poor-quality protein increases the risk of essential amino
26 acid (EAA) deficiency, particularly for lysine and threonine. Thus, it is necessary to be able to
27 detect easily EAA deficiency.

28 **Objective:** The purpose of this study was to develop metabolomic approaches to identify
29 specific biomarkers for an EAA deficiency, such as lysine and threonine.

30 **Methods:** Three experiments were performed on growing rats. In Experiment 1, rats were fed
31 for 3 weeks with lysine (L30), or threonine (T53) deficient gluten diets, or non-deficient
32 gluten diet (LT100) in comparison with the control diet (milk protein, PLT). In Experiments
33 2a and 2b, rats were fed at different levels of lysine (L) or threonine (T) deficiency: L/T15,
34 L/T25, L/T40, L/T60, L/T75, P20, L/T100 and L/T170. 24h urines and blood samples from
35 portal vein and vena cava were analyzed using LC-MS. Data from Experiment 1 were
36 analyzed by untargeted metabolomic and Independent Component - Discriminant Analysis
37 (IC-DA) and data from Experiments 2a and 2b by targeted metabolomic and a quantitative
38 PLS regression model. Each metabolite identified as significant by PLS or ICDA was then
39 tested by one-way ANOVA to evaluate the diet effect. Two phases linear regression analysis
40 was used to determine lysine and threonine requirements.

41 **Results:** ICDA and PLS found molecules that discriminated between the different diets. A
42 common metabolite, the piperolate, was identified in Experiments 1 and 2a, confirming that it
43 could be specific to lysine deficiency. Another metabolite, taurine, was found in Experiments
44 1 and 2b, so probably specific to threonine deficiency. Piperolate or taurine breakpoints
45 obtained gives a value closed to the values obtained by growth indicators.

46 **Conclusion:** Our results showed that the EAA deficiencies influenced the metabolome.
47 Specific urinary biomarkers identified could be easily applied to detect EAA deficiency and to
48 determine which AA is deficient.

49 **Keywords:** Metabolomic; Lysine deficiency; Threonine deficiency; Pipecolate; Taurine.

50

51 **1. Introduction**

52 The amount of amino acid (AA) requirements and subsequent recommendation of amino
53 acids intake has remained a controversial issue in nutrition over time and FAO/OMS
54 recommended to treat dietary amino acids as individual nutrients (1). In humans, twenty AA
55 are proteinogenic, and are constituents of proteins. Of these twenty AA, nine are considered
56 as essential AA, which means that there is no *de novo* synthesis by the organism, or that they
57 are synthesized at an insufficient rate to cover the requirements and must therefore be
58 provided in the diet (2–4). Among these EAA, two are considered strictly essential, because
59 they do not participate in transamination reactions, namely lysine and threonine.

60 In developing countries, there is a risk of deficiency in some EAA. In these countries, the
61 protein sources are mainly derived from cereals. In Africa, for example, the diet is mainly
62 based on the consumption of cereals, vegetables, roots and tubers (5). In India, the majority of
63 the population is vegetarian. Their main source of protein is found in cereals such as rice,
64 wheat and millet (6). However, these cereals contain proteins of poor quality, deficient in
65 some EAA. Lysine is the first limiting AA in humans because it is deficient in cereal (7), with
66 a content of 270 mg/g nitrogen in wheat versus 619 mg/g nitrogen in milk (8). The risk of
67 deficiency in some EAA is also worldwide, particularly in sensitive population such as
68 gestating women (9) and the elderly (10).

69 A protein can only be synthesized if all its constituent AA are available in sufficient quantity
70 (11). If one of the EAA is deficient in the diet, the proteosynthesis will be limited to the level
71 of the limiting EAA. It is important that all EAA be available in sufficient quantities, in order
72 to maintain an adequate protein synthesis. Thus, it is necessary to know precisely what the
73 requirements in these EAA are.

74 Different methods have been developed to estimate AA requirements. The need for each EAA
75 was first estimated by studying a nitrogen balance. More recently, methods using the infusion
76 of stable isotopes have been developed (12). The method of direct AA oxidation of the test
77 AA, for example ^{13}C -lysine, can be used, as can the oxidation of an indicator AA, often ^{13}C -
78 phenylalanine, depending on the supply of the amino acid under study (13). However, these
79 methods are complicated, require invasive protocols, and the resulting requirement values
80 show significant differences according to method used. For example, for lysine, the estimated
81 requirement with nitrogen balance is evaluated at $12 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, while it is around $40 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$
82 with the method of indicator AA oxidation (14,15). This is why no dietary
83 recommendations are published for amino acids. There is therefore an urgent need to develop
84 more precise and non-invasive approaches (14). The emergence of new technologies and the
85 "omics" sciences, such as metabolomics, seem promising for developing a new method of
86 measuring AA needs. Metabolome profiles of biological fluids have already been successfully
87 used as early biomarkers of individual metabolic sensitivity and metabolic dysfunction (16),
88 and also to determine nutrient requirement such as choline (17,18).

89 The aim of this study was to investigate the effect of protein quality and quantity on the
90 plasma and urinary metabolomes and to determine biomarkers of lysine and threonine
91 deficiency or sufficiency in urine using a model of growing rats fed with lysine or threonine
92 deficient diets during a three weeks period. The hypothesis was that AA deficiency could
93 induce the production of degradation metabolites in biological fluids that would disappear or
94 appear when the AA intake reaches the requirement, and in this way, these metabolites could
95 be used as biomarkers.

96 **2. Materials and Methods**

97 **2.1. Animals**

98 Experiments 1, 2a and 2b were performed on growing Wistar Han rats (HsdHan[®]: WIST,
99 Envigo, France), weighing 75-100 g (4 weeks old) in experiment 1, and 50-60 g (3 weeks old)
100 in Experiment 2a and 2b. The rats were housed in individual clear Plexiglas cages in a
101 controlled temperature room (22°C) with a 12-hour light/12-hour dark cycle (lights on from
102 00:00 to 12:00). These studies were approved by the Regional Animal Care and Ethical
103 Committee and conformed to the European legislation on the use of laboratory animals
104 (registration number: APAFIS#13436-2017122616504600). For these 3 experiments, rats
105 were fed with a standard diet for one week before being switched to their experimental diet.
106 During the experimental period, a calibrated meal of 4 g (58.2kJ) was given between 12:00
107 and 12:30 every day, and they then had *ad libitum* access to food between 12:30 and 9:00 the
108 next day. The calibrated meal was given to accustom the rats to quickly consuming a small
109 amount of food. Food intake body weight and nasoanal length (NAL) were measured daily
110 over the three weeks. In the 3rd week, each rat was placed during 48h in a metabolic cage (24h
111 of adaptation and 24h of measurement) to collect urine for the metabolomics analyses.

112 For the design of diets, we used two protein sources: an animal protein source, which is the
113 total milk proteins and a plant protein source, gluten protein, naturally deficient in lysine and
114 threonine. Gluten and milk proteins were analyzed for the determination of crude protein
115 content and the profile of amino acids of each protein source (supplemental Table 1). Free
116 amino acids were added to these protein sources in the diets at the level of the EAA
117 requirements (19) except for the deficient EAA studied. The amount of the lacking EAA was
118 replaced by alanine, as a neutral amino acid. Thus, the amount of the protein in the diet
119 determines the level of EAA deficiency. We chose to maintain protein source in diet to avoid
120 any negative effect of free amino acid, particularly on food intake. However, to control this
121 effect, we added a second control group (LT100, L100 and T100, respectively), containing the
122 lowest amount of protein and the highest amount of free amino acids at the level of EAA

123 requirements (19). Thus, L15 or T15 and L100 or T100 are equivalent, except that L100 and
124 T100 have the same amount of lysine and threonine as P20. The P20 is the control diet which
125 contains 193.5g/kg of crude proteins, which correspond to 15% of net proteins, corresponding
126 to the requirements for growing rats (19). All the diets are isoenergetic and isonitrogenic. Food
127 pellets were prepared using powder manufactured at the unit Sciences de l'Animal et de l'Aliment de
128 Jouy (SAAJ), INRAE, Animal Diets Experimental Facility, Jouy-en-Josas, France (
129 <https://doi.org/10.17180/MAQZ-V844>).

130 Experiment 1:

131 Twenty-four growing rats were divided into 4 groups (n = 6 rats/group). These 4 groups were
132 assigned for 3 weeks one of the 4 isocaloric experimental diets described in Table 1a. Diets
133 were composed of two different protein sources: gluten or milk proteins . Gluten is an
134 unbalanced AA protein, especially for lysine and threonine. Gluten covers only 24.5% of
135 lysine requirement and we added free lysine to set the deficiency level to 70% (*i.e.* covering
136 30% of lysine requirement). For threonine, gluten induced 47% threonine deficiency (*i.e.*
137 covering 53% of threonine requirement). Three diets were made with gluten: L30 diet,
138 deficient in lysine but supplemented in threonine to cover 100% of its requirement; T53 diet,
139 deficient in threonine but supplemented in lysine to cover 100% of its requirement; and
140 LT100: supplemented in lysine and threonine to cover 100% of their requirements. The
141 control diet was the P20, which contains 193.5g/kg of crude proteins, corresponding to 15%
142 of net proteins, which is required to cover the requirements for growing rats (19). However,
143 we supplemented the P20 with five amino acids (methionine, cysteine, tryptophan, threonine,
144 alanine) to reach the same levels of amino acids as in the gluten and milk protein (P20) diets.

145 Experiment 2a:

146 Sixty-four growing rats were divided into 8 groups (n =8 rats/group). They were fed for 3
147 weeks isocaloric diets containing different levels of lysine, namely 15, 25, 40, 60, 75, 100 or
148 170% of the theoretical lysine requirement necessary for growing rats (19) (from 85% to 0%
149 deficiency), comparatively to the control P20 (19), as described in Table 1b. The amount of
150 lysine deficiency was determined by the protein level in the diet (Table 1b), and all amino
151 acids except the test lysine were supplemented to the level of the theoretical requirement
152 necessary for growing rats.

153 Experiment 2b:

154 The same procedure as in Experiment 2a was done for different levels of threonine, namely
155 15, 25, 40, 60, 75, 100 or 170% of the theoretical threonine requirement for growing rats (19)
156 (from 85% to 0% deficiency), comparatively to the control P20 diet (19), as described in
157 Table 1c. The amount of threonine deficiency was determined by the protein level in the diet
158 (Table 1c), and all amino acids except the test threonine were supplemented to the level of the
159 theoretical requirement for growing rats.

160 **2.2. Urine and plasma sampling**

161 During the 24h of urine collection, rats were housed individually in metabolic cages. Rats
162 were fed *ad libitum* with their respective diets.

163 In Experiments 2a and 2b, at 11:00 on the last day of the study, rats were fed with 4-gram
164 pellet (58.2 kJ) of their test-diet, to standardize the energy intake, and two hours later, they
165 were anesthetized with Isoflurane. Blood was taken from the portal vein and from the vena
166 cava. The blood samples were collected on heparin, centrifuged (4°C, 3000 rpm, 10 min) and
167 the plasma was stored at -80°C until metabolomics analysis.

168 **2.3. Metabolomics sample preparation and liquid-chromatography mass** 169 **spectrometry (LC-MS) analysis of urine**

170 Untargeted metabolomic: Urine sample preparation: fifteen micro-liters of each thawed 24h-
171 urine sample were diluted with 985 μ l of milliQ water/acetonitrile (90/10, v/v). UPLC-HRMS
172 analysis was performed on an Acquity H-Class system coupled to a Q-TOF Synapt G2 Si
173 instrument (Waters Corporation, Milford, MA) in positive electrospray ionization mode
174 (ESI+) using an Acquity CSH C18 column (2.1×100 mm; 1.7μ m bead size; Waters). The
175 column temperature was 40°C and the eluents A and B were 0.01% formic acid in water and
176 0.01% formic acid in acetonitrile, respectively. The gradient was run at 0.5mL/min and
177 consisted of an isocratic elution for 1 min of 2% B, followed by an increase of B at a linear
178 rate to 88% at 12 min, then a re-equilibration for 1 min with 2% B and maintenance at 2% B
179 until 17min.

180 Raw data from Synapt G2 Si were pre-processed using MarkerLynx (MassLynx V4.1,
181 Waters) to obtain a list of detected molecules characterized by m/z and retention time (Rt).

182 Targeted metabolomic: Plasma and urine samples were analyzed by LC-HRMS on a Dionex
183 Ultimate 3000 (Thermo Fisher Scientific, San Jose, CA) ultra-performance liquid
184 chromatography (UPLC) system coupled with a Thermo Q Exactive Plus mass spectrometer
185 using two chromatographic conditions: C18 and HILIC columns, both with 2 ionization
186 modes (positive and negative), in order to broaden the metabolome coverage. Four data
187 matrices were therefore obtained. Metabolite redundancy was removed by choosing variables
188 with lower coefficients of variation (CV) and higher intensities in the quality control samples.
189 The following workflow was applied to all samples: On the C18 column, samples were
190 analyzed during a 16 minutes gradient with solvents A and B, H_2O and 0.1% formic acid; and
191 acetonitrile and 0.1% formic acid, respectively.

192 On the HILIC column, samples were analyzed during a 27minutes gradient with solvents A
193 and B, 16 mM ammonium formate, and acetonitrile and 0.1% formic acid, respectively.

194 All samples were analyzed in FullScan mode and some were also analyzed in MS/MS mode
195 to facilitate the annotation of compounds. A Quality Control (QC) pool of all samples was
196 injected every 5 samples so as to correct for instrumental variation during the assay sequence.
197 The mass spectra were collected using resolving power 35 000 Full Width at Half Maximum
198 (FWHM) for the theoretical mass to charge ratio (m/z) 200. Full scan mass spectra were
199 acquired in the 250 – 1200 m/z range. The ionization source parameters for positive and
200 negative ion modes were as follows: capillary temperature 285°C, spray voltage 3.5 kV,
201 sheath gas 60 (arbitrary units), auxiliary gas 20 (arbitrary units), probe heater temperature
202 370°C and S-Lens RF level was set at 45 v.

203 Data extraction from the raw data acquired in FullScan was done using XCMS. Data were
204 cleaned with visual verification of EIC (Extracted Ion Chromatogram) and variables present
205 in the blanks as well as poorly integrated or very noisy peaks were removed. Data were
206 normalized to the QC samples with Van Der Kloet normalization (20) using R[®] scripts, in
207 order to correct for instrument analytical drift. Variables with coefficients of variation greater
208 than 30% in the pools were removed. Finally, the selected variables were matched with the
209 laboratory's internal database containing over 1000 pure metabolites with their mass spectra
210 and retention times using the in-house LCMS tool of the W4M platform (21).

211 **2.4. LC-MS data processing and analysis**

212 For experiment 1, the mass spectra measured on urine, plasma from the vena cava and plasma
213 from the portal vein were first pretreated (normalization with Probability Quotient
214 Normalization (PQN)) (22) followed by Pareto-scaling (23), and then analyzed by
215 Independent Component Discriminant Analysis (ICDA) (24,25), which is a supervised
216 discrimination method (for more details see (26)).

217 For Experiments 2a and 2b, in addition to ICDA, a quantitative Partial Least-Squares (PLS)
218 regression model was used to relate the pretreated mass spectra to the Protein concentration.
219 Variables corresponding to biomarkers indicating protein deficiency were identified from the
220 loading weights calculated in each model.

221 The results showed that similar biomarkers were found using the two chemometric methods.
222 As the difference between the different groups were related to the level of AA in the diet, we
223 assumed there should be a correlation between this level and the level of any significant
224 biomarker. Hence only PLS results for these experiments will be presented. The applied
225 chemometric procedure has been described in detail in (26). Note that in Experiments 2a and
226 2b, the models were built with the 6 groups L15, L25, L40, L60, L75 and L100 (or T15, T25,
227 T40, T60, T75 and T100). The samples in the "extrapolated groups" L100 and L170 (or T100
228 and T170) (excess of lysine with respect to protein) were projected onto the PLS model space,
229 and their predicted value for lysine (respectively threonine) concentration was compared to
230 the corresponding experimental value, to see whether they follow the same model as the
231 "regular" groups.

232 For each discriminant metabolite identified by PLS, the effects of the diets were tested by
233 one-way ANOVA using R[®] scripts. Pairwise comparisons were performed with post hoc
234 Bonferroni tests for multiple comparisons. Differences were considered significant at $p <$
235 0.05.

236 In order to determine the growth requirements, we used a two-phase linear model on the
237 discriminant biomarkers to identify the breakpoint. The concentrations of the metabolites
238 were first corrected by the volume collected in the 24h and using a homemade script, based on
239 the work of M. Kato and co-workers (27). We approximated this two-phase linear model by
240 the following equation:

241 $[Biomarker]_{urine} = b \pm \log(1 + \exp^{-m*(\%intake - a)})$, where b is the ordinate at the
242 breakpoint; m is the slope before the breakpoint and a is the abscissa at the breakpoint. These
243 parameters were fitted by the least-squares method. On the basis of this model, an estimated
244 value of growth requirements can be predicted, and is given as mean \pm 2sd.

245 3. Results

246 3.1. Potential biomarkers of lysine and threonine in urines of rats fed a gluten diet 247 supplemented with lysine or threonine, or both (Experiment 1)

248 In order to determine biomarkers for gluten and lysine or threonine deficiency, an ICDA
249 model was applied. The L30 and T53 groups were separated from the controls along IC2
250 (Figure 1A), whereas no discrimination was observed between the two controls, P20 and
251 LT100.

252 A one-way ANOVA test was applied to the molecules with strong contributions to IC2 and
253 twelve molecules were considered to have significant intensity differences according to group
254 membership: 2-(2-Ethyl-4,5-dihydro-1H-imidazol-1-yl)ethanol-1 was greater in groups where
255 lysine or threonine was deficient in the diet; 2,4-Dimethyl-2-[(piperidine-1-
256 carbothioyl)sulfanyl] pentanoic acid was smaller in rats with the P20 diet; 6-methylpyridine-
257 3-carboxamide, imidazolepropionic acid, 4-phenoxybenzene sulfonic acid and L-alanine-L-
258 alanyl were higher when lysine was deficient in the diet; 3-Methyladenine, Hydroxyprolil-
259 proline and pipecolate were smaller in the lysine deficient diet; methylhypoxanthine and 6-
260 methoxy-pyridine-3-carboxylic acid were higher while taurine was smaller in the threonine
261 deficient diet (Supplemental Figure 1).

262 Figure 1

263

264 **3.2. Urinary and plasma biomarkers for lysine deficiency (Experiment 2a)**

265 For the urine, portal vein and vena cava samples, the data were projected onto the scores plots
266 of the PLS models. The two groups with the lower lysine content were separated from the
267 others along LV1 (Figure 2A). The discriminant variables were identified based on their
268 contributions to LV1 being greater than +/- 1 standard deviations in the QQ-plots.

269 For urine, the PLS method revealed that forty-five molecules were influenced by the diets.
270 After using an ANOVA test, thirty-six molecules were confirmed to have significantly
271 different intensities according to group membership, among which nineteen were amino acids
272 or derived from amino acids. The details on each molecule are reported in Table 2 and
273 Supplemental Figure 2.

274 The intensity of twenty of these molecules was smaller when the percentage of lysine in the
275 diet increased (which means that these biomarkers are less present when the lysine intake
276 increases), namely 1-methylnicotinamide, anthranilate, gluconate, 2-
277 hydroxyphenylpropanoate, L-glutamic acid, hydroxyisocaproic acid, isomaltose, L-alanine, L-
278 arginine, L-carnitine, L-histidine, L-methionine, L-threo-3-phenylserine, L-tyrosine, L-valine,
279 phosphoric acid, proline, sucrose and tryptophan and L-phenylalanine.

280 The intensity of sixteen others compounds was greater when the percentage of lysine
281 increased (which means that increasing the lysine intake contributes to the synthesis of more
282 of these biomarkers), namely 2-isopropylmalic acid, 4-pyridoxate, cadaverine,
283 dehydroascorbate, isocitrate, L-gulonolactone, L-leucine, L-lysine, N-N-N- trimethyllysine,
284 nicotinic acid, N-methyl-2-pyridone-5carboxamide, norvaline, oxoglutarate, N-alpha-
285 acetyllysine, piperolate, and uracil.

286 Interestingly, the piperolate is the only metabolite identified in both Experiments 1 and 2a,
287 which suggests that it is specifically indicative of lysine deficiency (Figure 2B). When L170

288 was excluded from statistical analysis (because of its very large variation in this group), we
289 observed that it is lower under severe lysine deficient diet (L15 and L25) compared to P20
290 group and increased when the lysine intake increased.

291 **Figure 2**

292 For the portal vein, the PLS method revealed that twenty-seven molecules were associated
293 with the diets. The ANOVA test confirmed that seventeen molecules had significantly
294 different intensities according to group membership. Cytosine, iso 16-0 lysoPC, L-lysine, L-
295 methionine, N-N-N-trimethyllysine, N-methyl-2-pyridone-5-carboxamide and pipercolate
296 were greater when lysine increased in the diet. On the contrary, 4-Methyl-2-oxovaleric acid,
297 L-carnitine, L-histidine, L-leucine, L-serine, L-threonine, L-tyrosine, N-acetylserotonin, o-
298 acetyl-l-carnitine and tryptophan were smaller when lysine increased in the diet. The details
299 on each molecule are reported in Table 2 and Supplemental Figure 3.

300 For the vena cava, the PLS method showed that twenty-nine molecules were discriminant for
301 the groups. The ANOVA test confirmed that twenty molecules had significantly different
302 intensities according to group membership. The details on each molecule are reported in
303 Table 2 and Supplemental Figure 4. Some molecules intensities were greater when lysine
304 increased in the diet: cytosine, betaine, L-lysine, L-methionine, mannose-6-phosphate, N-N-
305 N-trimethyllysine, nicotinamide, N-methyl-2-pyridone-5-carboxamide and pipercolate. Others
306 were smaller when lysine increased in the diet: 2-hydroxybutyrate, L-carnitine, L-histidine, L-
307 serine, L-threonine, o-acetyl-l-carnitine, succinic acid and tryptophan. For three molecules, no
308 clear variations appeared (18-0 Lyso PC, gluconate and L-leucine).

309 Interestingly, when the metabolite profile was compared between urine and plasmas from
310 portal vein and vena cava, only nine molecules were similar for all three compartments (Table
311 2). Among these metabolites, five were amino acids (L-histidine, L-lysine, L-methionine,

312 tryptophan, leucine). Lysine in blood and in urine was higher when the amount of lysine
313 intake increased, which is not surprising. However, the increase of methionine in blood and
314 the inverse variation in urine, and the decrease in L-histidine and tryptophan, suggest a
315 relationship between lysine intake and the metabolic utilization of these amino acids. Among
316 the other metabolites found in the three compartments, three of them arise from lysine
317 metabolism: L-carnitine, N-N-N- trimethyllysine and pipercolate. The intensity of
318 pipercolate, which is the only metabolite also found in experiment 1, was greater in blood and
319 urine as does N-N-N- trimethyllysine. On the contrary, L-carnitine was smaller when the
320 amount of lysine increases. N-methyl-2-pyridone-5 carboxamide, which is one of the
321 metabolic products of nicotinamide adenine dinucleotide (NAD) degradation, was higher in
322 urine, portal and vena cava blood when the lysine intake increases.

323

Table 2

324 **3.3. Urinary and plasma biomarkers for threonine deficiency (Experiment 2b)**

325 For the urine, portal vein and vena cava samples, the three lowest threonine diets were
326 separated from the others along LV1 (Figure 3). QQ-plots were used to select the most
327 relevant variables for the separation of groups.

328 For urine, the PLS method showed that twenty-six molecules varied with the diets. The
329 ANOVA test showed that all molecules had significantly different intensities according to
330 group membership. The details on each molecule are reported in Table 3 and Supplemental
331 Figure 5.

332 Among these molecules, fourteen had a smaller intensity when the percentage of threonine in
333 the diet increased, namely aspartate, beta-alanine, galactose, L-tyrosine, lactose, L-carnitine,
334 L-glutamic acid, L-phenylalanine, palatinose, pyridine-2-aldoxime methochloride, sucrose,
335 taurine, trehalose, and urate; while twelve others were higher when threonine increased,

336 namely 2-oxoadipate, 2-oxobutanoic acid, carnosine, cis-aconitate, citrate, dehydroascorbate,
337 glutarate, isovaleroylglycine, dimethyl oxalate, methylmalonic acid, N-methyl-2-pyridone-5-
338 carboxamide, oxoglutarate. Interestingly, taurine is the only metabolite identified in
339 Experiments 1 and 2b, which suggests that it is specifically indicative of threonine deficiency
340 (Figure 3).

341 **Figure 3**

342 For the portal vein, the PLS method showed that twenty-two molecules varied with the diet.
343 The ANOVA test confirmed that nine discriminated the diets. When threonine increased,
344 some molecules intensities were higher: L-serine, L-methionine, cytosine, N-acetylglycine
345 homoserine and citrate. Other molecules had smaller intensity when threonine increased:
346 pipecolate and L-arginine. kynurenic acid was also significantly affected by threonine content
347 of the diet but no clear variation could be seen. The details on each molecule are reported in
348 Table 3 and Supplemental Figure 6.

349 For the vena cava, the PLS method showed that twenty-four molecules were found
350 discriminant but only nine were shown to be significant by the ANOVA test (Table 3,
351 Supplemental Figure 7). When threonine increased, some molecules had higher intensity:
352 mevalolactone, aspartate, cytosine, N-acetylglycine, homoserine and L-methionine, whereas
353 other decreased: pipecolate, L-arginine and methylmalonic acid.

354 When the different molecules in urine and in plasmas from portal vein and from vena cava
355 were compared, only one molecule, citrate, was found to be significant in both plasma and
356 urine, and the metabolites found in portal vein and vena cava followed the same variations:
357 smaller for arginine and pipecolate and greater for L-methionine, L-serine, N-acetylglycine,
358 homoserine and cytosine.

359 **Table 3**

360 **3.4 Breakpoint identification of lysine and threonine requirements**

361 In order to investigate whether the urinary biomarkers identified for lysine and threonine
362 deficiency could be used for the determination of lysine and threonine requirements, a
363 mathematical model was used to determine the breakpoint in the pattern of variation of
364 pipecolate and taurine biomarkers comparatively to growth indicators (body weight and
365 nasoanal length (NAL)) (Figure 4). In brief, the variable tested should have a bimodal pattern:
366 a progressive increase or decrease until the requirement for the tested amino acid is met, and
367 then no further change in the concentration of the variable (28). A two-phase linear regression
368 analysis could be calculated to determine the 'breakpoint' which corresponds to the inflection
369 point. This approach was applied to the identified biomarkers and the results are shown in
370 Figure 4. When a two-phase linear regression was applied on the relative urinary pipecolate
371 concentration from Experiment 2a, a breakpoint was obtained on $45.26 \pm 17.11\%$ of the
372 theoretical lysine requirement (Figure 4) which corresponds to 3.79 ± 0.65 g of lysine.kg⁻¹ in
373 the diet. Interestingly, the breakpoint obtained for urinary pipecolate relative concentration is
374 close to the breakpoint obtained for NAL ($43.72 \pm 4.4\%$ of the theoretical requirement) whereas
375 for body weight, it was $37.38 \pm 6.37\%$ of the theoretical requirement. The breakpoint obtained
376 from the relative urinary taurine concentration from Experiment 2b was found to be $47.62 \pm$
377 10.27% of the theoretical threonine requirement (Figure 4) which corresponds to 2.91 ± 0.63 g
378 of threonine.kg⁻¹ of the diet. This value is similar to the breakpoint obtained for the NAL
379 breakpoint ($48.16 \pm 3.78\%$ of the theoretical requirement), whereas for body weight, it was
380 $53.26 \pm 4.75\%$ of the theoretical requirement.

381 

382 **4. Discussion**

383 This study addressed the consequences of three weeks restriction of two essential amino acids
384 (EAA), lysine or threonine, on 24-hour urine, and plasma from portal vein and vena cava. To

385 this end, three different experiments were performed. Quantitative metabolite analysis of
386 biological fluids, urine or plasma, identified metabolites associated with the EAA restriction
387 that may eventually serve as biomarkers of EAA sufficiency/deficiency, and on the other
388 hand, the profile obtained may provide information on the impact of EAA restriction on the
389 organism. In the present study, these metabolomics analyses revealed that a single EAA
390 restriction caused observable perturbation to the urinary and plasma metabolic profiles of rats.

391 First of all, a pilot study where growing rats were fed either a lysine deficient gluten diet
392 (L30), or a threonine deficient gluten diet (T53), or a gluten control diet with 100% lysine and
393 threonine requirement (LT100), as well as a second control group with a diet with milk
394 proteins (P20) was performed. In this study, using non-targeted metabolomics, biomarkers
395 specific of EAA deficiency were identified. Pipecolate, 3-methyladenine and hydroxyprolil-
396 proline decreased for the lysine deficient group compared to threonine deficient group and
397 control, and taurine had a smaller intensity and 6-methoxy-pyridine-3-carboxylic acid had a
398 greater intensity for threonine deficient group compared to the other 3 groups. A gluten
399 specific biomarker was also identified: 2,4-Dimethyl-2-[(piperidine-1-carbothiyl)
400 sulfanyl]pentanoic acid was higher for the LT100, L30 and T53 groups. In Experiments 2a
401 and 2b, young growing rats were fed with diets differing in their percentage of lysine or
402 threonine, including very low lysine/threonine diet (L/T15, L/T25), moderately low (L/T40,
403 L/T60), adequate (L/T75, P20, L/T100), or above the requirement (L/T170) (19).

404 Metabolomics analyses on portal vein, vena cava and urine revealed that the majority of
405 metabolites found are AA or AA metabolites, and the discriminant molecules found in
406 Experiments 2a and 2b were different, except for N-methyl-2-pyridone-5-carboxamide, which
407 was greater in urine and blood for the lysine deficiency experiment and only in urine for the
408 threonine deficiency. Shibata and Matsuo proposed that the ratio between N1-methyl-2-
409 pyridone-5-carboxamide (2-pyr) and N1-methyl-4-pyridone-3-carboxamide (4-pyr)/N1-

410 methylnicotinamide (MNA), might be useful as an index to assess the adequacy of amino acid
411 intake in rats (29). However, in our study, we did not detect N1-methyl-4-pyridone-3-
412 carboxamide (4-pyr)/N1-methylnicotinamide (MNA). In lysine deficiency but not in threonine
413 deficiency, 1-Methylnicotinamide and Nicotinamide was detected in plasma but not in urine.
414 Thus, the hypothesis of Shibata and Matsuo was not verified in our experiments. Moreover,
415 we have already detected MNA in urines of protein deficiency which suggests that it not
416 specific to the deficiency of one amino acid (26).

417 Severe lysine deficiencies have an impact on protein or other AA metabolisms showing lower
418 levels of L-histidine, tryptophan, L-valine, tryptophan and proline, and higher level of L-
419 leucine as compared to the control group. It has been shown that in the case of an AA
420 deficient diet, the deficient AA is spared and its elimination in urine is reduced (30). These
421 metabolomic analyses revealed also an impact on the lysine metabolism pathway. Indeed,
422 trimethyllysine and N-alpha-acetyllysine were smaller when lysine decreased in the diet.
423 Trimethyllysine is a precursor of carnitine, explaining that carnitine is lower when the lysine
424 level is decreased in the diet (31). Studies have also shown that rats fed with a high lysine diet
425 present a higher concentration of trimethyllysine in skeletal muscle and in plasma compared
426 to control rats (32). Three others metabolites from AA metabolism were found to predict
427 lysine deficiency, L-threo-3-phenylserine, anthranilate, and pipercolate. L-threo-3-
428 phenylserine and anthranilate increase in severe lysine deficient diets.

429 The pipercolate is the only metabolite identified in Experiments 1 and 2a which suggests that it
430 is specifically indicative of lysine deficiency. The KEGG pathway for lysine degradation in
431 rats and mammals showed that the major route is the saccharopine pathway (Figure 5). This
432 mitochondrial pathway includes nine enzymatic reactions leading to the production of two
433 acetyl CoA. However, the literature review highlights the existence of the pipercolate pathway
434 in mammals, which is less characterized (Figure 5)(33). This pathway includes 4 enzymatic

435 processes: the α -transamination or α -deamination of lysine to 2-oxo-6-aminocaproic acid; the
436 spontaneous conversion of 2-oxo-6-aminocaproic acid into Δ^1 -piperideine-2-carboxylate
437 (P2C); the conversion of P2C to pipercolic acid; and the further conversion of pipercolate into
438 Δ^1 -piperideine 6-carboxylate (P6C). The pipercolic acid and saccharopine pathways then
439 converge at the level of L-2-aminoadipic acid 6-semialdehyde (AASA). AASA may undergo
440 spontaneous conversion into P6C and the latter can be converted into L-pipercolic acid.

441 Figure 5

442 The existence of the pipercolate pathway in mammals is supported by a review of the
443 literature. Using L- $[\alpha\text{-}^{15}\text{N}]$ lysine in α -aminoadipic semialdehyde (α -AASA) dehydrogenase-
444 deficient fibroblast, Struys et al. 2010 have shown the alternative pathway of pipercolate
445 formation (35). Moreover, this catabolic pathway may be the main route for lysine
446 degradation in brain (33). Pipercolate is also an indicator of peroxisomal dysfunctions (36).
447 Whether the intake of dietary lysine modulates the pipercolate synthesis and impact
448 peroxisomal biogenesis remains to be investigated.

449 For the effect of threonine deficiency, as in a lysine deficient diet, some monosaccharides
450 were found in urine for threonine deficient diets, namely sucrose, palatinose, lactose,
451 galactose and trehalose, which may arise from urine contamination by foods. Some AA and
452 related metabolites have a modification of their intensities according to threonine level in the
453 diet: Aspartate, Beta-alanine, L-glutamic acid, L-tyrosine, L-phenylalanine and taurine were
454 smaller in urine when threonine increases in the diet, while L-methionine, L-serine and
455 homoserine were higher in both portal and vena cava blood. L-carnitine was higher under T15
456 and differs significantly from the other groups. An impact of Krebs cycle metabolites is also
457 observed for threonine deficient diets. In urine, some intermediates, such as oxoglutarate, cis-
458 aconitate and citrate, increase proportionally to threonine level in the diet. Interestingly,
459 citrate is the only molecule found to be significant in plasma and urine. Some studies on food

460 restriction or protein energy malnutrition have already shown that there is a decrease in Krebs
461 cycle intermediates (37,38). Urate increased in severe and moderate threonine deficient diets
462 (T15, T25 and T40) and decreased from T60 to P20. It is a degradation product of purine, a
463 metabolite present in protein diet, that is regularly detected in urine (39). Some other
464 metabolites in urines were lower for threonine deficient diets, such as carnosine, glutarate,
465 isovaleroylglycine, 2-oxoadibate, 2-oxobutanoic acid, which are molecules of the AA
466 degradation pathways, and N-methyl-2-pyridone-5-carboxamide and dehydroascorbate.
467 Among these metabolites, taurine is the only one that was also identified in Experiment 1,
468 which suggests that it is a good candidate to indicate a threonine deficiency. In our previous
469 work on protein restriction biomarkers (26), taurine in urine was not found to be significant.
470 However, bile salts such as taurocholic acid that results from the conjugation of cholic acid
471 with taurine, is found in portal vein under low protein diet. AA promoted taurocholic acid
472 production (40) would explain the decrease in taurocholic acid under low protein/AA diet. An
473 important step for bile production is conjugation with the amino acids, taurine or glycine, that
474 occurs in the liver. In rats and human, there are glycine and taurine bile acid conjugates and
475 rats excrete mostly taurine conjugates (41). Interestingly, a hypocholesterolemia effect of
476 wheat gluten was reported in rats, with an increased excretion of bile acids (42). However, the
477 supplementation of wheat gluten with lysine or threonine did not reverse this effect. Gut
478 bacteria are also involved in biotransformation of bile salts during their enterohepatic
479 circulation. The hydrolysis of conjugated bile acids releases free bile acids and glycine or
480 taurine (43). Indeed, these data suggest that there is a relation between threonine, taurine and
481 bile synthesis. However, further studies are needed to clarify this metabolic pathway. We
482 hypothesize that during threonine deficiency, taurine could be more used to synthesized
483 taurocholic acid and the variation of taurine or taurine salt in urine depends of the microbiota
484 profile.

485 However, unlike threonine, taurine is synthesized from cysteine (and thus methionine too)
486 (44–46). The sulfur AA metabolic pathways are linked to threonine (Figure 6). Thus, the
487 production of taurine is dependent on the presence of these AAs. Gluten, a diet deficient in
488 threonine (T53), which decreases taurine, contains twice as many sulfur AAs as milk protein,
489 which could explain the inverse variations observed between the two experiments in our
490 studies, an increase in Experiment 1 and a decrease in Experiment 2b. Interestingly, Faure et
491 al observed an increase in taurine plasma concentration in rats fed diet meeting 30% of their
492 threonine requirements for growth, which is in accordance with the variations observed in
493 Experiment 2b. Moreover, as for us, Faure et al did not find significant differences in plasma
494 taurine between diets covering 60, 100, and 150% of the rats threonine requirements for
495 growth (47). This link between threonine and taurine remains to be clarified.

496

497

Figure 6

498 In order to investigate whether the urinary biomarkers identified for lysine and threonine
499 deficiency could be used for the determination of lysine and threonine requirements, we used
500 a two phases linear regression analysis to determine the breakpoint. Commonly, this method
501 has been used to determine amino acid requirements with the indicator amino acid oxidation
502 method (IAAO), and the 24h-indicator amino acid oxidation and balance method (24h-
503 IAAO/IAAB) (28). Our results showed that the breakpoint obtained on the basis of pipercolate
504 or taurine gives a value of $45.26 \pm 17.11\%$ and $47.62 \pm 10.27\%$ of the theoretical requirement,
505 respectively. Interestingly, the value of the requirement obtained on the basis of the NAL as
506 growth indicators was in fact close to the value determined using urinary biomarkers ($43.72 \pm$
507 4.4% and $48.16 \pm 3.78\%$, for lysine and threonine, respectively). This suggests the official
508 requirements for these two amino acids (19) were overestimated. The final values for
509 requirements estimated by urinary biomarkers were 4.58 ± 1.73 and 2.91 ± 0.63 g./kg⁻¹ of the

510 diet and by NAL 4.43 ± 0.45 and 2.94 ± 0.23 g/kg⁻¹ of the diet for lysine and threonine,
511 respectively. Interestingly, the same value (0,45% of lysine content of the diet for maximum
512 body weight gain) was obtained by Ishibashi and Kametaka (49) using ¹⁴C recovery in
513 respiratory CO₂ in rats injected by L-Lysine-U-¹⁴C and by body weight gain methods. Thus,
514 these results highlight that the biomarkers identified by metabolomics could be used to
515 determine amino acid requirements. However, further studies are required to estimate
516 interindividual variations to allow dietary recommendation.

517 In summary, in these three experiments, with non-targeted metabolomics (Experiment 1) and
518 targeted metabolomics approach (Experiments 2a and 2b), two biomarkers were identified:
519 pipecolate and taurine. Pipecolate is lower for low lysine diets (Experiment 1 and 2a), but not
520 for low threonine diets. Moreover, an earlier work of our team on protein restriction
521 biomarkers (26) also indicated that pipecolate in urine was lower for low protein diets. These
522 results seem to indicate that pipecolate is specific to lysine deficiency. Pipecolate is a
523 metabolite of the lysine degradation pathway. The decrease of lysine in the diet induced a
524 decrease in its degradation explaining its lower concentration for severe lysine deficient diets.
525 Taurine is the second biomarker found in two experiments on protein/AA deficiency.
526 Interestingly, taurine does not present the same variation between these two experiments but it
527 discriminates between the groups and differentiates the threonine deficient diets from the
528 control diets. Further studies are required to confirmed whether taurine is specific to threonine
529 deficiency.

530 **5. Conclusion**

531 To conclude, we identified two biomarkers specific to EAA deficiency in rats: pipecolate for
532 lysine and taurine for threonine. Additional experiments are needed to clarify the effect of
533 threonine on taurine excretion in urine. These two biomarkers, which can predict the state of
534 sufficiency in lysine or threonine in rats in experimentally controlled conditions, are

535 promising. Their measurement in the urine could make it possible to know the AA status of an
536 individual, and thus to detect a possible state of deficiency.

537

538 **6. Patents**

539

540 **Author's contributions:** Daniel Tomé, Dalila Azzout-Marniche – **Designed research;**

541 Joanna Moro, Gaëtan Roisné-Hamelin, Nadezda Khodorova, Douglas N. Rutledge, Jean-

542 Charles Martin, Catherine Tardivel – **Conducted research;** Joanna Moro, Gaëtan Roisné-

543 Hamelin, Pierre Barbillon, Delphine Jouan-Rimbaud Bouveresse, Dalila Azzout-Marniche –

544 **Analyzed data or performed statistical analysis;** Joanna Moro, Gaëtan Roisné-Hamelin,

545 Delphine Jouan-Rimbaud Bouveresse and Dalila Azzout-Marniche – **Wrote paper;** Delphine

546 Jouan-Rimbaud Bouveresse and Dalila Azzout-Marniche – **Had primary responsibility for**

547 **final content.**

548

549 All authors have read and approved the final manuscript.

550

551 Data described in the manuscript, code book, and analytic code will be made available upon

552 reasonable request.

553

554 **7. References**

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689 **FIGURES CAPTIONS**

690 **Figure 1. Lysine and threonine potential biomarkers in urines of rats fed a gluten diet**
691 **supplemented with threonine or lysine or both. (A)** ICDA scores plot for the LC-MS data,
692 characterizing the discrimination of the predefined groups in urine of rats fed a gluten-based
693 diet. **(B)** Urinary pipecolate and taurine relative intensities variations. 24 rats were fed during
694 3 weeks a gluten diet (L30, T53 or LT100) or a control diet (P20). In the 3rd week, each rat
695 was placed during 48h in a metabolic cage (24h of adaptation and 24h of measurement) to
696 collect urine for the metabolomics analyses. Data were analyzed by untargeted metabolomic
697 and IC-DA. The results of Pipecolate and Taurine, the potential biomarkers of lysine and
698 threonine deficiency, respectively, were represented by box plot (n=6 per group). Data that
699 do not share the same letter are different at the $p < 0.05$ level. L30: gluten diet deficient in
700 lysine but supplemented in threonine to cover 100% of its requirement; T53 diet, gluten diet
701 deficient in threonine but supplemented in lysine to cover 100% of its requirement; LT100:
702 gluten diet supplemented in lysine and threonine to cover 100% of their requirements; P20:
703 control diet.

704

705 **Figure 2: Pipecolate, a potential urinary biomarker for lysine deficiency in rats.** PLS
706 analysis for the LC-MS data, characterizing the discrimination of the predefined groups to
707 discriminate lysine content diet based on LV1 in urine (A) and urinary pipecolate variation
708 among the level of lysine intake (B). 64 rats were fed during 3 weeks different levels of lysine
709 (L) deficiency diets: 15, 25, 40, 60, 75, 100 or 170% of the theoretical lysine requirement
710 necessary for growing rats (19) or a control diet (P20) (n=8 by group). 24h urines were
711 collected and analyzed by targeted metabolomic and quantitative PLS analysis. The sample
712 indexes correspond to the sample number in our analyzed data matrix. The results of

713 Pipecolate, a potential urinary biomarker for lysine deficiency, were represented by box plot
714 (n=8 per group). Data that do not share the same letter are different at the $p < 0.05$ level.

715 **Figure 3: Taurine, a potential urinary biomarker for threonine deficiency in rats.** PLS
716 analysis for the LC-MS data, characterizing the discrimination of the predefined groups to
717 discriminate threonine content diets, based on LV1 in urine (A) and urinary taurine variations
718 for the different levels of threonine intake (B). 64 rats were fed during 3 weeks different levels
719 of threonine (T) deficiency diets: 15, 25, 40, 60, 75, 100 or 170% of the theoretical threonine
720 requirement necessary for growing rats (19) comparatively to the control diet (P20) (n=8 by
721 group). 24h urines were collected and analyzed by targeted metabolomic and quantitative PLS
722 analysis. The results of Taurine, a potential urinary biomarker for threonine deficiency, were
723 represented by box plot (n=8 per group). Data that do not share the same letter are different at
724 the $p < 0.05$ level.

725

726 **Figure 4. Breakpoint identification of lysine and threonine requirements.** The results of
727 urinary taurine and pipecolate, body weight and nasoanal length (Experiments 2a and 2b)
728 were analysed by two-phase liner model using the following equation: $[Biomarker]_{urine} =$
729 $b \pm \log(1 + \exp^{-m * (\%intake - a)})$, where b is the ordinate at the breakpoint; m is the slope
730 before the breakpoint and a is the abscissa at the breakpoint. The estimated value for
731 requirements corresponds to the mean value and the recommendation is given as mean \pm 2sd.

732

733 **Figure 5. Lysine degradation pathway.** The KEGG lysine degradation pathway (34), in red,
734 shows that saccharopine pathway is the only pathway for the degradation of lysine in rats, and
735 more generally in mammals. An alternative pathway, the pipecolate pathway, in blue, have
736 been also described also in mammals (33). The pipecolic acid and saccharopine pathways

737 then converge at the level of L-2-aminoadipic acid 6-semialdehyde (AASA). AASA may
738 undergo spontaneous conversion into P6C and the latter can be converted into L-pipecolic
739 acid.

740 **Figure 6. Threonine degradation pathway.** The KEGG threonine degradation pathway (48)
741 active in rats, and more generally in mammals, is represented in green. Rather than threonine,
742 taurine is synthesized from cysteine and methionine. The sulfur AA metabolic pathways are
743 linked to threonine. Thus, the production of taurine is dependent on the presence of these
744 AAs. The degradation pathway of threonine to taurine is indicated in blue.

745

746

747 **Table 1.** (A) **Detail of the diet design of experiment1.** Two protein sources were used: milk
748 and gluten proteins. Free amino acids were added to these protein sources in the diets at the
749 level of the EAA requirements (19) except for the deficient EAA studied. The amount of the
750 lacking EAA was replaced by alanine, as a neutral amino acid. P20 and LT100 are the
751 controls. L30: gluten diet supplemented in EAA to cover 100% of their requirements except
752 for lysine, LT100: gluten diet supplemented in EAA to cover 100% of their requirements,
753 P20: milk protein diet, T53: gluten diet supplemented in EAA to cover 100% of their
754 requirements except for threonine.

	L30	T53	LT100	P20
Amino acid content (g/kg)				
Threonine	2.92	-	2.92	0.52
Lysine	0.47	6.91	6.91	-
Isoleucine	1.42	1.42	1.42	-
Leucine	1.95	1.95	1.95	-
L Cystine	2.30	2.30	2.30	3.96
Histidine	0.18	0.18	0.18	-
Methionine	2.91	2.91	2.91	1.29
Tryptophane	0.80	0.80	0.80	0.21
Valine	2.26	2.26	2.26	-
Alanine ^a	6.44	2.92	0	6.69
Weight content (g/kg)				
Proteins				
From Gluten ^b	194.55	194.55	194.55	-
From Milk protein ^c	-	-	-	193.5
Added free amino acids	21.66	21.66	21.66	12.67
Starch	555.98	555.98	555.98	564.64
Sucrose	90.51	90.51	90.51	91.92
Soy oil	40	40	40	40
Minerals	35	35	35	35
Vitamin	10	10	10	10
Cellulose	50	50	50	50
Choline	2.3	2.3	2.3	2.3
Total (g/kg)	1000	1000	1000	1000
Energy content (%)				
Protein	23	23	23	22
Carbohydrate	66	66	66	65
Fat	11	11	11	11
Energy density (kJ/g)	15	14.95	14.87	14.77

755
756 ^aFree alanine was added to adjust the total of amino acids between diets. All the diets were
757 isonitrogenous and isoenergetic.

758 ^bGluten protein content was 77.6% (Crude proteins)

759 ^cMilk protein content was 77.1% (Crude proteins)

760

761

763 **Table 1. (B) Detail of the diet design of experiment 2a.** In experiment 2a, rats were fed
 764 isocaloric diets containing different levels of lysine, namely 15, 25, 40, 60, 75, 100 or 170%
 765 of the theoretical lysine requirement necessary for growing rats (19), comparatively to the
 766 control P20. The amount of lysine deficiency was determined by the protein level in the diet,
 767 and all amino acids except the test lysine were supplemented to the level of the theoretical
 768 requirement necessary for growing rats. L: Lysine

	L15	L25	L40	L60	L75	P20	L100	L170
Free amino acids added (g/kg)								
Histidine	2.8	2.5	2	1.3	0.8	-	2.8	-
Isoleucine	5.3	4.7	3.8	2.5	1.6	-	5.3	-
Leucine	11.5	10.2	8.2	5.4	3.4	-	11.5	-
Lysine	-	-	-	-	-	-	8.6	7.2
Threonine	5.2	4.6	3.7	2.4	1.5	-	5.2	-
Tryptophan	2.5	2.2	1.8	1.2	0.7	-	2.5	-
Valine	6.7	5.9	4.8	3.2	2	-	6.7	-
Arginine	4.4	3.9	3.1	2.1	1.3	-	4.4	-
Methionine	2.9	2.6	2.1	1.4	0.9	-	2.9	-
Phenylalanine	5.6	4.9	3.9	2.6	1.6	-	5.6	-
Alanine	23.7	22.2	20	17	14.8	-	15.1	-
Total other NEAA	57.6	51	40.8	27.2	17	-	57.6	-
Weight content (g/kg)								
Milk proteins ^a	29	48	77	116	145	193.5	29	193.5
Added free amino acids	128.3	114.7	94.1	66.3	45.7	-	128.3	7.2
Starch	576.4	576.4	576.4	576.4	576.4	576.4	576.4	576.4
Sucrose	92.8	92.8	92.8	92.8	92.8	92.8	92.8	92.8
Soy Oil	40	40	40	40	40	40	40	40
Minerals	35	35	35	35	35	35	35	35
Vitamins	10	10	10	10	10	10	10	10
cellulose	50	50	50	50	50	50	50	50
choline	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
Water	36.2	30.75	22.4	11.2	2.8	-	-	-
Total (g/Kg)	1000	1007.2	1000	1000	1000	1000	1000	1000
Energy content (%)								
Protein + free AA	19.8	19.8	19.8	19.9	19.9	20	19.8	20.2
Carbohydrate	69.1	69	68.9	68.8	68.8	68.5	69.1	68.2
Fat	10.1	10.2	10.2	10.3	10.3	10.4	10.1	10.5
Energy density (kJ/g)	15	14.95	14.87	14.77	14.77	14.57	15	14.45

769 ^aMilk protein content was 77.1% (crude proteins)

771 **Table 1. (C) Detail of the diet design of experiment 2b.** In experiment 2b, rats were fed
 772 isocaloric diets containing different levels of threonine, namely 15, 25, 40, 60, 75, 100 or 170%
 773 of the theoretical threonine requirement necessary for growing rats (19), comparatively to the
 774 control P20. The amount of threonine deficiency was determined by the protein level in the
 775 diet, and all amino acids except the test threonine were supplemented to the level of the
 776 theoretical requirement necessary for growing rats. T: Threonine.

	T15	T25	T40	T60	T75	P20	T100	T170
Free amino acids added (g/kg)								
Histidine	2.8	2.5	2	1.3	0.8	-	2.8	-
Isoleucine	5.3	4.7	3.8	2.5	1.6	-	5.3	-
Leucine	11.5	10.2	8.2	5.4	3.4	-	11.5	-
Lysine	8.6	7.6	6.1	4.1	2.5	-	8.6	-
Threonine	-	-	-	-	-	-	5.2	4.3
Tryptophan	2.5	2.2	1.8	1.2	0.7	-	2.5	-
Valine	6.7	5.9	4.8	3.2	2	-	6.7	-
Arginine	4.4	3.9	3.1	2.1	1.3	-	4.4	-
Methionine	2.9	2.6	2.1	1.4	0.9	-	2.9	-
Phenylalanine	5.6	4.9	3.9	2.6	1.6	-	5.6	-
Alanine	20.3	19.2	17.6	15.4	13.8	-	-	-
Total other NEAA	57.6	51	40.8	27.2	17	-	57.6	-
Weight content (g/kg)								
Milk proteins	29	48	77	116	145	193.5	29	193.5
Added free amino acids	128.35	114.77	94.12	66.31	45.66	0	128.34	4.3
Starch	576.4	576.4	576.4	576.4	576.4	576.4	576.4	576.4
Sucrose	92.8	92.8	92.8	92.8	92.8	92.8	92.8	92.8
Soy Oil	40	40	40	40	40	40	40	40
Minerals	35	35	35	35	35	35	35	35
Vitamins	10	10	10	10	10	10	10	10
cellulose	50	50	50	50	50	50	50	50
choline	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
water	36.2	30.75	22.4	11.2	2.8	0	36.2	0
Total (g/Kg)	1000	1000	1000	1000	1000	1000	1000	1004.3
Energy content (%)								
Protein + free AA	20.2	20.3	20.5	20.7	20.9	20	20.2	20
Carbohydrate	68.3	68.2	68.1	67.9	67.7	68.5	68.3	68.5
Fat	9.3	9.3	9.3	9.3	9.3	9.3	9.3	9.3
Energy density (kJ/g)	14.60	14.62	14.65	14.70	14.73	14.57	14.60	14.57

777

778 ^aMilk protein content was 77.1% (crude proteins)

779 **Table 2: Discriminant metabolites in urine, plasma of portal vein and of vena cava for lysine deficiency.** Rats were fed at different levels of
 780 lysine (L) deficiency diets: 15, 25, 40, 60, 75, 100 or 170% of the theoretical lysine requirement necessary for growing rats (19) comparatively to
 781 the control diet (P20) (n=8 by group). Plasma from portal and vena cava and 24h urines were collected and analyzed by untargeted metabolomic
 782 and quantitative PLS analysis. Data are presented as changes from L15 to L170: red represents an increase, green a decrease and grey, no clear
 783 variations. White cells indicate that the metabolite was not detected. For more details, see Supplemental Figures 2, 3 and 4.

	VP	VC	Urine
AA			
L-Alanine			Green
L-Arginine			Green
L-Glutamic_acid			Green
L-Histidine	Green	Green	Green
L-Leucine	Green	Grey	Red
L-Lysine	Red	Red	Red
L-Methionine	Red	Red	Green
L-Phenylalanine			Green
L-Serine	Green	Green	
L-Threonine	Green	Green	
L-tyrosine	Green		Green
L-Valine			Green
Proline			Green
Tryptophane	Green	Green	Green

Food			
2-Hydroxyphenyl Propanoate			Green
iso-Maltose			Green
Sucrose			Green

	VP	VC	Urine
AA metabolism			
L-Carnitine	Green	Green	Green
o-acetyl-l-carnitine	Green	Green	
2-Hydroxybutyrate		Green	
L-Threo-3-Phenylserine			Green
4-Methyl-2-Oxovaleric acid	Grey		
N-N-N-Trimethyllysine	Red	Red	Red
N-Alpha-Acetyllysine			Red
N-Acetylserotonine	Green		
Pipecolate	Red	Red	Red
Hydroxyisocaproic acid			Green
Anthranilate			Green
Betaine		Red	

Krebs intermediate			
succinic acid		Green	

Lipid metabolism			
iso-16-0_LysoPC	Red		
iso-18-0_LysoPC		Grey	

	VP	VC	Urine
Carbohydrate metabolism			
Mannose 6-Phosphate		Red	
other			
Cytosine	Red	Red	
N-methyl-2-pyridone-5-carboxamide	Red	Red	Red
1-Methylnicotinamide			Green
Dehydroascorbate			Red
Isocitrate			Red
Gulonolactone			Red
Nicotinamide		Red	
Nicotinic_acid			Red
Oxoglutarate			Red
Phosphoric acid			Green
4-Pyridoxate			Red
Uracil			Red
Microbiota			
Cadaverine			Red
2-isopropylmalic acid			Red
Gluconate		Grey	Green
Norvaline			Red

784 **Table 3: Discriminant metabolites in urine, plasmas of portal vein and vena cava for threonine deficiency.** Rats were fed at different levels
 785 of threonine (T) deficiency diets: 15, 25, 40, 60, 75, 100 or 170% of the theoretical threonine requirement necessary for growing rats (19)
 786 comparatively to the control diet (P20) (n=8 by group). Plasma from portal and vena cava and 24h urines were collected and analyzed by
 787 untargeted metabolomic and quantitative PLS analysis. Data are presented as changes from T15 to T170: red represents an increase, green a
 788 decrease and grey, no clear variations. White cells indicate that the metabolite was not detected. For more details, see Supplemental Figures 5, 6
 789 7.

	VP	VC	Urines
AA			
L-arginine			
Aspartate			
Beta-alanine			
L-Glutamic acid			
L-tyrosine			
L-Methionine			
L-Phenylalanine			
L-Serine			

Krebs cycle metabolite			
Oxoglutarate			
Cis-aconitate			
Citrate			

	VP	VC	Urines
AA metabolism			
N-Acetyl glycine			
L-carnitine			
Carnosine			
Glutarate			
Homoserine			
Isovaleroylglycine			
Kynurenic acid			
2-oxobutanoic acid			
2-Oxoadipate			
Pipecolate			
Taurine			

Fat metabolism			
Methylmalonic acid			

	VP	VC	Urines
Food			
Dimethyl oxalate			
Glactose			
Lactose			
Palatinose			
Pyridine-2-aldoxime			
Sucrose			
Threalose			
Other			
Cytosine			
Dehydroascorbate			
N-methyl-2-pyridone-5-carboxamide			
Methylmalonic acid			
Mevalolactone			
Urate			

790

791